Author's response to reviews

Title: Modulation of Expression of Heat Shock Proteins and Apoptosis by Flueggea leucopyrus (Willd) decoction in three breast cancer phenotypes.

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Author's response to reviews: see over
Executive Editor,
BMC Complementary and Alternative Medicine,

Dear Sir,

Our manuscript titled ‘Modulation of Heat Shock Proteins and Apoptosis by *Flueggea leucopyrus* (Willd) decoction: possible mechanisms mediating cytotoxicity to three breast cancer phenotypes’ has been submitted for publication as a research article in your highly esteemed journal, BMC Complementary and Alternative Medicine.

In view of the urgency of discovering natural agents that can target breast cancers based upon specific molecular subtypes, this article demonstrates the inhibitory effect of the decoction prepared from *F. Leucopyrus* (aerial parts) on the proliferation of specific phenotypes of breast cancer cells, via inhibition of Heat Shock Proteins and enhancement of apoptosis. Thus *F. leucopyrus* could be further exploited to develop cheaper and natural alternatives to existing drugs that would not only be useful for the treatment of breast cancer, but could also prevent disease relapse. *F. leucopyrus* can easily be cultivated without incurring much expenditure and, development of an anti-cancer agent from this plant would also be of economic benefit to breast cancer patients worldwide.

We declare that this manuscript is original, has not been published before and is not currently being considered elsewhere. We also wish to confirm that there is no competing interest and the manuscript has been read and approved by all the named authors.

We feel these findings will be of interest to the readers of your journal and we hope you find our manuscript appropriate for publication and look forward to hearing from you.

Yours Sincerely,

Anuka Mendis
Replies to the reviewers reports: 2

I sincerely thank the reviewer’s for their comments and advice, and I have taken it in to consideration. I have made the necessary changes, and below I have replied to the comments.

Reviewer: R Narayanan

Major compulsory revisions:

1) DNA fragmentation analysis: No clear fragmentation observed. The figure is unacceptable in the present form. Support this with another set of data. Paclitaxel should also be included. Also, provide at least a single set of data for the concentrations used for other experiments. Include MCF-10A.

A new set of data has been provided with regard to DNA fragmentation. Cells treated with Paclitaxel has been included. MCF10A treated with the decoction has also been included. See figure 6.

2) With no confirmatory experiments to related HSP expression and apoptosis, it cannot be stated as “possible mechanism” in the manuscript title. Results of SKBR cells contradict the hypothesis explained between HSP expression and Apoptosis. There are no clear explanations for this. As we stated earlier, the novelty of the current study is the mechanism explained. Hence the relation between HSP and apoptosis should be explained in some form. At least, include references relating HSP expression and apoptosis.

The title has been changed to “Modulation of Expression of Heat Shock Proteins and Apoptosis by Flueggea leucopyrus (Willd) decoction in three breast cancer phenotypes”.

The relation of HSP expression and apoptosis has been explained from line 353 to 420.

3) MCF-10A should be included in HSP expression analysis and apoptosis.

MCF10A has been included in quantitative immunofluorescence for HSP expression (Fig 4) as well as DNA fragmentation (Fig 6).
Reviewer: Andy Göbel

Major Compulsory Revisions

1. “It’s not mentioned by the authors, why Fig. 1 contains Paclitaxel as a positive control, but none of all the other experiments. The treatment with Paclitaxel should at least be included in the assessment of DNA-fragmentation or Acridine orange/Ethidium bromide staining to compare the effectiveness of the decoction treatment.”

The author didn’t respond to this sufficiently. Again, it is not clear, why experiments with paclitaxel as a positive control were performed in Fig. 1 but not in all the other figures. The rationale for using paclitaxel in this study should be mentioned and explained. As the author want to link a reduced HSP70/90 expression with apoptosis by the decoction, one would expect, that the authors used paclitaxel since it has been shown already, that this substance also reduces HSP70/90. Otherwise it doesn’t make sense to include paclitaxel at all. In addition, to support the hypothesis that a reduced HSP70/90 expression is linked with apoptosis, the author should have included a more suitable positive control that is already known for its inhibitory effect on HSPs thereby exerting anti-tumor effects in cancer cells. If not further explained, using paclitaxel as a positive control in a study about linking HSP70/90 and apoptosis in cancer cells still lacks a reasonable rationale.

A new set of data has been provided with DNA fragmentation and the three breast cancer cells treated with paclitaxel has been included. (Fig 6)

Line 247 – 251, 383-390, 410-415 explains the use of paclitaxel in this study.

We were unable to purchase a suitable positive control for HSP’s as the cost was beyond what was available in the research grant. However the use of paclitaxel and its relations between HSP70/90 and apoptosis has been explained in line

2. “Fig. 5 doesn’t contain any legend which should be included since it is not explained which lane contains which treatment. Additionally, also this figure should contain at least one positive control (e.g. Paclitaxel). Furthermore the concentrations that were used for this experiment should be comparable to those that were used for Acridine orange/Ethidium bromide and HSP70/HSP90 staining. A DNA fragmentation with 400 µg/ml of decoction and more could also
be a non-specific effect by overloading the cells with the isolated plant proteins. This is of importance to possibly link decreased HSP70/90 expression and induced loss of membrane integrity with induction of apoptosis by 20 µg/ml or 40 µg/ml decoction.” The author also didn’t respond to this point sufficiently. As they were able to show a loss of cell survival and an activation of caspases as well as a positive AO/EB staining 24 h after the treatment with concentrations up to 100 µg/ml, it is still confusing that they exceeded these concentrations and the incubation time for DNA fragmentation assay. Apoptosis always results in the fragmentation of DNA. Hence, the used concentrations for the caspase activation assay should be high enough to achieve an obvious effect. Moreover, the authors show gene expression of HSP70 and HSP90 upon the treatment with 10 µg/ml and 20 µg/ml, but not for the concentrations that were used for the DNA fragmentation assay. It is not predictable if the HSP70/90 expression follows the same pattern 48h after treatment with these high concentrations as shown for lower concentrations in a shorter incubation time. Nonetheless an incubation time of 48h would be acceptable upon using the same conditions as in the other experiments.

Legend of fig 5 is now fig 6 is present in line 639.

This comment has been taken in to consideration and we have performed DNA fragmentation again with Paclitaxel positive control. Cells were treated with 40 µg/ml and 100 µg/ml and for 24hrs. Please see fig 6

3. In line with the suggestions of Reviewer 1 and evaluating the results of this study at a glance, the author should be more careful with their conclusion, that a reduced expression of HSP70/90 by the decoction induces apoptosis in the breast cancer cell lines. It should be further discussed, that it may not only be one bioactive compound of the decoction but rather a synergistic effect of different compounds that are exerting these effects. One possible scenario could be that one component reduces HSP70/90 activity in the cancer cells thereby facilitating an increased pro-apoptotic effect of any other – so far unknown – component. Nonetheless, the discussion of the contradictory results in SKBR-3 cells is acceptable.

The statement has been modified, please refer line 391-395

4. Finally, comparable to paclitaxel, it is not clear, why MCF-10A cells as a non-cancerous breast cell line was just included in figure 1 but in none of the other experiments.
MCF10A has now been included in quantitative immunofluorescence for HSP expression (fig 4) as well as DNA fragmentation (fig 6).

Minor Essential Revisions

1. Line 233 contains a mistake: “…has significant cytotoxic 24h incubation= 28.6μg/ml)
   This has been corrected line

2. No reference mentioned in line 269: “In breast cancer cells, overexpression of HSP90 and HSP70 are reported to correlate with poor prognosis.”
   The respective reference has been added.

3. If not indicated so far, please mention that the isolation and identification of the bioactive compound(s) of the decoction is necessary.
   This has been included in line 417-420.